

Evaluation of housekeeping genes for normalizing RT-qPCR and analysis of the expression patterns of WRKY1 transcription factor and rhynchophylline biosynthetic-related genes in *Uncaria rhynchophylla*

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Abstract

Background: *Uncaria rhynchophylla* Miq. Miq.ex Havi, a traditional medicinal herb, is enriched with a number of pharmacological active terpenoid indole alkaloids (TIAs). At present, a comprehensive selection and evaluation of the appropriate housekeeping genes for gene expression analysis, especially transcription factors and key enzyme genes involved in biosynthesis pathway of TIAs in *U. rhynchophylla*, have not been reported. Reverse transcription quantitative PCR (RT-qPCR) is currently the most common method for gene expression level detection with its high sensitivity, specificity, reproducibility, and ease of use. However, this methodology is dependent on the selection of an optimal housekeeping gene for the accurate normalization of RT-qPCR results.

Results: Ten candidate housekeeping genes, that are homologs of genes used in other plant species as common housekeeping genes, were used to evaluate their expression stability under three stress related experimental treatments (methyl jasmonate, ethylene and low temperature), using multiple stability analysis methodologies. The results showed that S-adenosylmethionine decarboxylase (*SAM*) had a higher expression stability than the other candidate housekeeping genes under the experimental conditions tested. Using *SAM* as a housekeeping gene, 14 genes of key TIA enzymes and a *WRKY1* transcription factor had their expression profiles examined in the three experimental stress treatments that are known to affect the accumulation of TIAs in *U. rhynchophylla*. The expression pattern of *WRKY1* was found to be similar that of tryptophan decarboxylase (*TDC*) and strictosidine- β -D-glucosidase (*SGD*).

Conclusions: This research is first to report the stability of housekeeping gene in *U. rhynchophylla* and as such provides an important foundation for future gene expression analysis in *U. rhynchophylla*. *WRKY1* expression indicated it is potentially capable of coordinating the expression of *TDC* and *SGD*, providing a possible means of enhancing alkaloid production in future with synthetic biology.

1 Background

Uncaria rhynchophylla (Miq.) Miq.ex Havi is evergreen lianas, belonging to the *Rubiaceae* family [1], has been used to treat convulsions, hypertension, epilepsy, eclampsia and other cerebral diseases for ages as a traditional Chinese herbal medicine [2]. *U. rhynchophylla* contains alkaloids, flavonoids, triterpenoid and other active ingredients [3–5]. The main active ingredients in *U. rhynchophylla* are terpenoid indole alkaloids (TIAs), such as rhynchophylla (RIN) and isorhynchophylla (IRN), which have a significant effect in the treatment the Alzheimer's disease by acting on multiple pathological processes [6]. Therefore, *U. rhynchophylla* TIAs pathways has been the focus of many studies related to its potential value to the pharmaceutical industry.

Although its active ingredients have many therapeutic properties the major challenge for their utilization by the pharmaceutical industry is the low production rate of these alkaloids [7], and the fact that genetic improvement through traditional breeding is long and costly. TIAs such as RIN and IRN are thought to be part of a plants chemical defense against pest, and stress signal-related molecules or pathogen-derived elicitors are often used to induce the accumulation of these precious pharmaceutic active metabolites [8]. However, with the advent of synthetic biology, research is now centered on the molecular elucidation of the important biosynthesis pathways of medicinal plants such as *U. rhynchophylla*, so as to identify key genes that synthesize and control alkaloid content that can allow these alkaloid pathways to be established and expressed in more genetically amenable systems [9].

TIAs biosynthesis involves multiple and complex metabolic pathways. All TIAs are derived from tryptamine and secologanin, derived from the tryptophan and iridoid terpene biosynthesis pathways, respectively. The biosynthetic pathway for RIN and IRN are shown in Fig. 1. In the first committed step of iridoid terpene biosynthesis, Geranyl-PP is converted to geraniol by the geraniol synthase (GES) and a variety of functional enzymes participate in oxidation, reduction, glycosylation and methylation reactions. Finally, Secologanin is generated by oxidative cleavage of Loganin by the enzyme secologanin synthase (SLS). In the tryptamine pathway, Chorismate is catalyzed by a variety of enzymes to generate L-tryptophan which is catalyzed by tryptophan decarboxylase (TDC). Thus, the condensation of secologanin and tryptamine, catalyzed by strictosidine synthase (STR), forms strictosidine. Finally, it is desugared by strictosidine- β -D-glucosidase (SGD).

With the development of high-throughput sequencing technology, many biosynthesis pathways of medicinal plants have been elucidated. Quantitating and validating gene expression profiles by reverse transcription quantitative real-time PCR (RT-PCR) is one of the more important methods to identify functional genes [10], as well as its role in microbial assays, and single nucleotide polymorphism determination [11, 12], owing to its high sensitivity, high specificity and simple operation [13]. However, the accuracy and reliability of the results is dependent on a number of factors including the quality of RNA, the efficiency of reverse transcription, the specificity of the primers and the stability of expression of the house-keeping genes that is used to normalize RNA levels between samples [14, 15]. Recent studies have shown that the stable expression of house-keeping genes in different tissues, organs, developmental periods and under different treatment conditions is rare or nonexistent [16, 17]. Traditional house-keeping genes commonly used for RT-PCR in plants include glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Actin (*ACT*), elongation factor 1 α (*EF-1 α*), 18S ribosomal RNA (*18S rRNA*), α -tubulin (*TUA*), and S-adenosylmethionine decarboxylase (*SAM*) but they are not always stably expressed in all tissues or conditions [16, 17]. Thus, systematic experimental design for the selection of stable house-keeping genes is an important first step towards applying RT-PCR in new plant species. Obtaining a validated housekeeping gene will enable the investigation of potentially important genes such as the *WRKY1* transcription factor (TF), and key enzyme genes in the TIAs pathways in *U. rhynchophylla*.

In this research, ten candidate house-keeping genes *ACT6*, Cyclophilin (*CYP*), *SAM*, *TUA*, *18S*, *EF-1 β* , Protein phosphatase 2A (*PP2A*), *GAPDH*, Phenylalanine Ammonia lyase (*PAL*), Cell division control protein 73 (*cdc73*) of *U. rhynchophylla* were selected based on their use as housekeeping genes in other plant species and genome data from third generation high-throughput sequencing platform Oxford Nanopore Technologies. Three stress related treatments methyl jasmonate (MeJA), Ethylene (ETH) and low temperature, were used to assay the expression level by RT-PCR in leaves of *U. rhynchophylla*, and analyzed for genes displaying stable expression profiles. Upon selection of the most suitable house-keeping genes the expression pattern of the "upstream pathway genes" related to the synthesis of TIAs and the transcription factor *WRKY1*, which has been reported to positively regulate the TIAs in some plants [18] were analyzed in the three treatments. Our work lays the foundation for the future functional analysis of key genes associated with the biosynthesis and regulation of TIAs pathways.

2 Results

2.1 Screening of candidate housekeeping genes, and primer-specific analysis

In this study, ten candidate housekeeping genes were selected from based on having been used for normalization in RT-qPCR analyzes in other plant species, and based on expression profiles based on our genome Nr database of *U. rhynchophylla* (unpublished). Amplification efficiency (E) and correlation coefficients (R^2) were calculated by a standarad curve with a series of five different cDNA dilutions. The candidate housekeeping gene abbreviation and names, primer sequences, amplification efficiency (E) and correlation coefficient (R^2) are listed in the Table 1. Primer specificity of the candidate housekeeping genes were checked by RT-qPCR. As showed in Supplementary Fig. 1, the melting curves of the candidate housekeeping genes all showed a single and smooth melting peak and the repeatability of PCR amplification curves was good. The amplification efficiency ranged from 99.3% for *ACT6* to 105% for *EF1-β*, the correlation coefficient of 10 candidate housekeeping genes was above 0.99.

Table 1
Primer sequence and genes of RT-qPCR for ten candidate house-keeping genes of *U. rhynchophylla*

Gene abbreviation	Gene name	Primer Sequence 5'-3' (F/R)	Amplification efficiency (%)	R^2
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	TACCACCAACTGTCTTGCTCTT CGCCTCTCCAGTCCTTCATT	99.7	0.991
<i>CYP</i>	Cyclophilin	CGAGAAAGGCGTGGGAAAG TGAGACCCGTTGGTGTGG	102.7	0.998
<i>PP2A</i>	Protein phosphatase 2A	CGCTGATGTTCTACGTCTACCTAAT AGTAGTAACGGTCCTCGGCTATA	99.8	0.996
<i>SAM</i>	S-adenosylmethionine decarboxylase	CACAATCTGGCATAACGAAA AACTCACTTGGCTGGAAAC	102.8	0.998
<i>TUA</i>	α-Tubulin	TCCCTTCTTGAGCACACTGAT CCATCAAACCTCAAAGACGCA	101.7	0.995
<i>ACT6</i>	Actin 6	ACCGAGCGTGGTTATTCTT TTCCTGCTGCTTCCATTCC	99.3	0.995
<i>PAL</i>	Phenylalanine Ammonia lyase	ATCGCTGAATCCTCCAATA CCACCCTACTCCACAATACTT	102.8	0.993
<i>EF1-β</i>	Elongation factor 1 beta	AAGGCATCCACCAAGAAGA AAGGCAACAATGTCACAGC	105.0	0.996
<i>cdc73</i>	Cell division control protein 73	TGGTGGCTGTTTTTCGTGTT TGATGCCGCTTATTCTTGC	100.2	0.995
<i>18S</i>	18S Ribosomal RNA	CTTCGGGATCGGAGTAATGA GCGGAGTCCTAGAAGCAACA	102.4	0.999

2.2 Expression abundance of the candidate housekeeping genes

The Ct value directly reflects the gene expression abundance (when amplicons have similar reaction efficiency), and the smaller the Ct value, the higher the gene expression abundance. In order to evaluate stability of the housekeeping genes in all treated experimental samples, the expression abundances of the ten candidate housekeeping genes were measured by the mean Ct values. As indicated by the Fig. 2, the Ct values ranged from 8.39 for *18S* to 27.55 for *GAPDH* in the three treatments in all samples, and majority of were distributed between 22 and 25. The *18S* gene had the lowest Ct values and *GAPDH* had the highest values, indicating that *18S* had the highest expression in all samples, whereas *GAPDH* had the lowest in all samples. The box-plot not only displays the expression abundance of the housekeeping gene, but also gives an indication of the variation of expression, because each housekeeping gene had different coefficients of variation, the lower values represent less variability. *SAM* showed the least variability, with a SD value of 0.17, followed by *CYP*, *PP2A* and *18S*. Whereas, *EF1-β* showed the highest mean Ct value (22.56 ± 0.48). However, to ensure that the reference gene is stable their expression was analyzed using different algorithms.

2.3 Expression stability analysis of candidate housekeeping genes

TIAs such as RIN and IRN production is known to respond pest and stress signal-related molecules. Therefore, it is necessary when selecting for a gene with stable gene expression that this is also analyzed under relevant abiotic stresses conditions. In order to gauge a genes potential as a housekeeping for analyzing genes involved in TIA pathways, three stress related experiments were performed involving low temperature, ETH, and MeJA (see Material and methods), to ensure that expression was not affected these conditions. To analyze the housekeeping genes under these three treatments, four algorithms;

GeNorm, NormFinder, Bestkeeper, and Delta Ct were used to verify their expression stability. The RefFinder online website was performed to systematically select the optimal housekeeping genes under all the different experimental conditions.

2.3.1 GeNrom analysis

For GeNrom the original Ct values of the genes were converted into relative expression Q (see Material and methods), and then the M value was determined. The M value reflects the expression stability with a low M value, indicating higher expression stability. As shown in Fig. 3, the candidate housekeeping genes had different stability under the three treatments. The M values of *SAM* and *PP2A* were the smallest in three treatments and thus identified as the most stable of the genes. *EF-1 β* and *18S* also showed good stability under MeJA. The *cdc73* gene was as stable as *SAM* under ETH. The stability of *TUA* was lower than other candidate housekeeping genes under three treatments, indicating that this gene was not useful for normalization purposes in this tissue under these conditions. GeNorm can also determine the appropriate number of housekeeping genes by calculating the pairwise variation ($V_n/n + 1$). If $V_n/V_n + 1 < 0.15$, the optimal number of suitable housekeeping genes is n . Conversely, the optimal number of housekeeping genes is $n + 1$. In this study, all results of pairwise variation under three treatments calculated by GeNorm were less than 0.15 (Fig. 4), suggesting that two housekeeping genes can be used for normalization.

2.3.2 NormFinder analysis

Similar to the analysis method of GeNorm, the original Ct values of the genes were converted into the relative expression Q according to the formula, and then the M value of the ten candidate housekeeping genes was calculated with M value negatively correlated with expression stability, i.e., smaller M values have higher stability, and so more suitable for the normalization. Table 2 shows that the ranking of 10 candidate genes as calculated by the NormFinder algorithm. Among the MeJA, ETH, 'control', and 'total' treatment subsets, it is observed that *SAM* was the most stable with the highest score. Under the low temperature treatment (0.104), *PP2A* was identified as the most stable gene, with *SAM* (0.260) the second most stable. Similar to the GeNorm analysis, *TUA* had the least stable expression.

Table 2
Expression stability rank of ten candidate housekeeping using
NormFinder in *U. rhynchophylla*

Rank	MeJA	ETH	Low-temperature	Control	Total
1	SAM 0.291	SAM 0.203	PP2A 0.104	SAM 0.145	SAM 0.507
2	PP2A 0.303	ACT6 0.282	SAM 0.260	PP2A 0.210	cdc73 0.605
3	ACT6 0.310	cdc73 0.427	ACT6 0.290	CYP 0.212	CYP 0.722
4	cdc73 0.334	EF1- β 0.443	cdc73 0.346	18S 0.237	ACT6 0.745
5	18S 0.434	CYP 0.547	GAPDH 0.541	GAPDH 0.253	PAL 0.813
6	PAL 0.513	PP2A 0.653	18S 0.542	TUA 0.273	PP2A 0.816
7	EF1- β 0.573	GAPDH 0.665	CYP 0.807	PAL 0.277	EF1- β 0.965
8	CYP 0.621	PAL 0.674	PAL 0.865	cdc73 0.333	GAPDH 0.965
9	GAPDH 0.753	18S 0.958	EF1- β 1.097	ACT6 0.341	18S 1.289
10	TUA 1.194	TUA 0.967	TUA 1.413	EF1- β 0.521	TUA 1.403

2.3.3 BestKeeper analysis

BestKeeper, unlike GeNorm and NormFinder, can directly analyze the Ct values obtained by RT-qPCR and calculate the standard deviation (SD) and coefficient of variation (CV) of Ct values to determine the expression stability of candidate housekeeping genes. The software uses SD = 1 as the threshold value, if SD > 1, the expression is unstable and not suitable for normalization. Whereas the smaller the SD, the more stable the expression of the gene. The CV reflects the variation of the expression level of the genes in different treatments, with the smaller the CV, the more stable expression. The results of BestKeeper analysis are shown in Table 3. In the ETH, low temperature, 'control', and 'total' treatment subsets, *SAM* was ranked as the most stable. Under MeJA treatment, *ACT6*

(1.32 ± 0.31) was considered the most stable, then *cdc73* (1.40 ± 0.35), with *SAM* (1.48 ± 0.33) as the third most stable. The least stable reference genes calculated by BestKeeper was *TUA* and *18S*. Genes with SD > 1 such as *TUA*, indicated low expression stability in our conditions and therefore are not suitable for normalization.

Table 3
Rank of ten candidate housekeeping genes in *U. rhynchophylla* (coefficient of variation (CV) ± standard deviation (SD))

Rank	MeJA	ETH	Low-temperature	Control	Total
1	ACT6 1.32 ± 0.31	SAM 1.55 ± 0.35	SAM 1.95 ± 0.50	SAM 0.62 ± 0.14	SAM 1.98 ± 0.46
2	<i>cdc73</i> 1.40 ± 0.35	ACT6 2.01 ± 0.44	PP2A 2.06 ± 0.51	PP2A 0.87 ± 0.22	<i>cdc73</i> 2.17 ± 0.54
3	SAM 1.48 ± 0.33	<i>cdc73</i> 2.28 ± 0.57	<i>cdc73</i> 2.29 ± 0.53	CYP 0.88 ± 0.18	ACT6 3.03 ± 0.69
4	PP2A 1.55 ± 0.36	PAL 2.36 ± 0.55	ACT6 2.45 ± 0.55	PAL 0.94 ± 0.22	PP2A 3.38 ± 0.78
5	EF1-β 1.67 ± 0.25	PP2A 3.09 ± 0.51	GAPDH 2.66 ± 0.42	TUA 1.02 ± 0.22	EF1-β 3.40 ± 0.75
6	PAL 1.70 ± 0.38	GAPDH 3.16 ± 0.77	18S 2.95 ± 0.27	<i>cdc73</i> 1.04 ± 0.26	CYP 3.80 ± 0.75
7	GAPDH 2.67 ± 0.65	CYP 3.20 ± 0.62	EF1-β 2.97 ± 0.68	GAPDH 1.12 ± 0.17	GAPDH 3.82 ± 0.96
8	CYP 3.15 ± 0.63	EF1-β 3.25 ± 0.69	PAL 3.12 ± 0.75	ACT6 1.26 ± 0.29	PAL 4.26 ± 0.67
9	18S 3.27 ± 0.30	TUA 3.74 ± 0.76	CYP 3.66 ± 0.74	EF1-β 1.85 ± 0.42	TUA 5.36 ± 1.15
10	TUA 4.24 ± 0.94	18S 7.96 ± 0.87	TUA 5.23 ± 1.14	18S 2.02 ± 0.18	18S 9.17 ± 0.89

2.3.4 Delta Ct analysis

Gene expression stability was assessed by calculating the mean standard deviation (SD) of Ct values of the ten candidate housekeeping genes. The smaller the mean SD, the higher the expression stability. As shown in the Supplementary Fig. 2, the results are consistent with previous analysis. *SAM* was found to be more stable in the three treatments than the other candidates and *TUA* was the least stable gene under all experimental conditions. Hence, *SAM* was found to be the most qualified gene to use for normalization under the conditions tested.

2.3.5 RefFinder analysis

RefFinder analysis is a method for comprehensive stability evaluation by assigning appropriate weights to the results of each candidate housekeeping gene in each program, and then calculating the geometric mean of the stability value weights of all algorithms for overall ranking. The RefFinder analysis method avoids the one-sidedness of individual algorithms and provides a comprehensive analysis of the expression stability of candidate housekeeping genes. The smaller the stability value, the better the stability of the gene. As shown in the Supplementary Table 1, *SAM* showed the most stable in all treatments except for MeJA (where it was fourth), consistent with the results of the GeNorm, NormFinder, Bestkeeper, Delta Ct algorithms. Above statistical methods, *SAM* ranks first in each algorithm, however, *PP2A* doesn't always come in second (according to GeNorm, *SAM* and *PP2A* were applied for normalization). So, *SAM* were used as housekeeping gene for RT-qPCR.

2.4 Expression patterns of WRKY1 and selected genes involved in TIAs pathway

Screening a large number of candidate TIA biosynthetic genes by "genome+ transcriptome + metabolome" co-expression association analysis, this model is highly predictable for the screening of multiple genes or super gene family candidates, and can greatly reduce the scope of subsequent gene research. We identified 22 types of genes in the biosynthesis pathway of *Siraitia grosvenorii* Mogroside V using transcriptome data, and screened 7 CYP450 and 5 UDPG candidate genes from 80 CYP450 and 90 UDPG by combining "transcriptome + expression profile + content" co-expression analysis [19]. One or two of these genes were validated by prokaryotic expression and eukaryotic expression in yeast [20, 21]. Co-expression pattern screening of priority candidate genes is a significant reference for improving the efficiency of screening super gene family target genes of other species. Taking the STR gene as an example (Supplementary Fig. 1), a total of 22 STR candidate genes were identified from the genome, and by transcriptome and content co-expression analysis, three sequences were found to be clustered with RIN and IRN content, from which the g3541 gene was firstly selected as the focus sequence for qRT-PCR analysis because the clustering relationship nearest and full-length gene.

From the Genome Nr database, only one gene was found to be annotated as *WRKY1*. *SAM* was used as the housekeeping gene to identify and validate *WRKY* expression and potential key TIA biosynthetic genes whose expression alters with the application of MeJA, ETH and cold stress. We performed qRT-PCR on leaves from tissue culture seedlings that were treated by MeJA and ETH at 0, 1, 2, 4, 8, 24 h, and low-temperature treatments at 0, 4, 8, 12, 24 and 48 h. Under MeJA treatment, *WRKY1* was significantly up-regulated at 4 h (Fig. 5a), then started to decrease by 8h and returned to the near original expression levels by 24 h. Expression patterns of *TDC*, *SGD* were similar to the *WRKY1*, especially the expression of *TDC* in response to MeJA at 4 h which increased 3.4 fold compared to 0 h. Some genes such as *STR* and *SLS*, which were up-regulated at 8 h, remained higher than untreated at 24 h. The genes, *G8H* and *8-HGO* expression decreased starting at 1 h after MeJA treatment, then decreased by 4 hours and then gradually return to original levels. As shown in the Fig. 5b, *WRKY1* also displayed significant changes in expression in response to ETH, which was similar to its response to MeJA. Compared to 0 h *WRKY1* was up-regulated 3 fold by 4 h, then started to decrease by 8 h. The expression of *TDC* was similar to the expression pattern of *WRKY1*, as it increased 4 hours after treatment, and then slowly declined. The highest gene expression of *STR* and *SGD* was at 8 h after treatment, but returned to almost initial expression levels at 24 h. The cold stress treatment differed from the other two treatments (Fig. 5c). *WRKY1* transcript levels remained lower until 24 h, then rose at 48 h. Among these genes, only the *TDC* gene had an expression pattern similar to *WRKY1*. For most genes, their expression increased by 4 h and then decreased.

Overall the expression profile of *WRKY1* was similar with that of *TDC* and *SGD* under the three treatments. This could imply that *WRKY1* is possibly involved in coordinating their expression which could enhance alkaloid production.

3 Discussion

In recent decades, the protective effects of *U. rhynchophylla* and its major components, TIAs, on the central nervous system and treatment of cardiovascular diseases have become a major research focus [22, 23]. As the major active therapeutic ingredients derived from *U. rhynchophylla* are RIN and IRN, understanding and identifying the unique genes involved in their biosynthesis and regulation are of great significance [24]. Therefore, it has become important to be able to quantitate the expression of known and candidate genes associated with TIA biosynthesis in *U. rhynchophylla* with the specificity, sensitivity, and ease that RT-qPCR provides. However, this methodology is dependent on the knowledge of stable housekeeping genes for normalization, which requires careful empirical determination in the tissues and conditions relevant for the genes and species investigated.

Although the medicinally active content in the leaves of tissue culture seedlings is not as high as that found in naturally occurring *U. rhynchophylla* plants, it is a more renewable and sustainable resource, and can produce higher biomass, making it a attractive source tissue for RIN and IRN [25, 26]. Therefore, our research focused on tissue culture seedlings leaves to evaluate the stability of housekeeping genes for this research. The stability of housekeeping genes was analyzed under stress-related signaling molecules MeJA, ETH, and cold stress that is known to affect TIA production. Cold stress is also a significant threat for plant productivity and impacts on plant distribution and crop production, particularly when it occurs during the growth phase [27].

A number of previous studies have indicated the expression levels of many traditional so-called housekeeping genes can vary among different species, tissues and change under different experimental conditions in the same species [28–31]. At present, there is no report on the identification of a gene with a stable gene expression profile under different stress treatments, suitable for RT-pPCR normalization in *U. rhynchophylla*. In this research, we studied ten potential candidate housekeeping genes that were selected based on gene homologs that have been used as normalization genes in other plant species. These included protein synthesis (*18S*), organelle skeleton (*ACT*, *TUA*), biological metabolic processes (*SAM*, *EF-1 β* , *PP2A*, *GAPDH*) and protein folding (*CYP*), regulation of plant growth (*CDC73*, *PAL*). In *Petunia hybrida*, *CYP* was found as the most stable gene during flower and leaf developments [32]. In *Brachypodium distachyon*, the expression of *SAM* ranked as the most stable in plants grown under various environmental stresses (high salt/drought), and *GAPDH* also plays a housekeeping role in *Brachypodium* growth and development [33]. In *Orchardgrass*, *ACT* and *CYP* were determined as the best reference genes for ABA studies [34]. In *Tibetan hulless barley*, *TUA* and *EF-1 β* were the most suitable reference genes under cold stress, and *ACT* was the most stable under drought stress [35]. In *Baphicacanthus cusia*, *18S* was found to be most stable gene under ultraviolet irradiation and hormonal stimuli (MeJA and ABA) [36]. In *Sinobambusa tootsik* f. *luteoalbostrata*, *SAM* was the most stable internal reference gene among the four leaf colors [37].

The raw Ct values of candidate housekeeping genes are a direct readout of expression level, the lower the Ct value, the higher the gene expression level, and a narrow Ct variation range implies high stability [38, 39]. As shown in Fig. 1, the Ct values of candidate housekeeping genes range from 8.4 to 27.6 under our experimental conditions and majority of them were distributed between 22 and 25. Moreover, *EF-1 β* and *GAPDH* had wide Ct variation ranges, indicating they are not be suitable as housekeeping genes for our work. In order to comprehensively evaluate and compare the expression stability of the candidate housekeeping genes, different software and algorithms (GeNorm, NormFinder, Bestkeeper, Delta Ct) were used. According to our overall analysis *SAM* ranked as the most consistent, but there are some differences between analyzes. For example, under cold stress condition, *SAM* was ranked top by GeNorm and Bestkeeper, however it was ranked second by NormFinder. In the MeJA treatment subset, *SAM* was the most stable housekeeping gene using NormFinder, but was ranked second, third or fourth position in Delta Ct, Bestkeeper or GeNorm respectively. This apparent divergence is probably due to the nature of the different software and their different weights placed on different sources of variation [40]. GeNorm identified two reference genes with the highest degree of similarity in expression profile and the lowest intra-group variation [41, 42]. The NormFinder software has been described to be less robust with small sample sizes compared to the GeNorm algorithm [43]. For Bestkeeper, the standard deviation (SD) and coefficient of variation (CV) of the Ct values were calculated to determine the expression stability of the candidate housekeeping genes. The RefFinder website is a popular tool for housekeeping gene verification, because it is free and performs a quick analysis using the three algorithms for housekeeping gene validation starting from a single input of the Ct values only [44], and then calculates the mean of weights of each gene by every algorithm [45]. The lower geomean rank implies that the housekeeping gene is more stable. *SAM* was identified as the most stable housekeeping gene for *U. rhynchophylla* in our experiments. Combining the analysis of several algorithms, *SAM* is the most stable than any other housekeeping genes under all treatments overall. In addition, the stability of *PP2A*, *cdc73* were found to be good and only slightly lower than that of *SAM* in some algorithms, so that they may be used as an additional reference gene for qRT-PCR under similar experimental conditions in *U. rhynchophylla*. Specifically, we found that *TUA* and *EF-1 β* were relatively unstable with their expression in our experiments.

U. rhynchophylla produces a large array of TIAs, yet they are generally produced at low production rates [3]. Many studies have shown that TIAs are defense molecules in response to biotic and abiotic [46]. The biosynthesis pathway of TIAs in *U. rhynchophylla* is a multistep, branched pathway [24]. A number of genes encode the ~20 enzymes involved in the biosynthesis pathway of TIAs. However, heterologous expression of several rate-limiting key enzyme genes has not resulted in satisfactory production levels of TIAs. Recently there have also been reports that ectopic expression of transcription factors can enhance alkaloid production [47]. Specific TFs can coordinate the transcription of multiple biosynthesis pathway genes, making them effective in metabolic engineering [18]. Some studies have shown that the W-box (TTGACC/T) is a cognate binding site for WRKY TFs. In this study, we found that the expression pattern of *TDC* and *SGD* is similar to that of observed for *WRKY1* under three stress treatments. This could imply that *WRKY1* is possibly involved in their regulation. Analysis of the promoter sequences of *TDC* and *SGD* using the PlantCARE (<http://sphinx.rug.ac.be:8080/PlantCARE/>) software to predict their promoters elements showed that *TDC* and *SGD* have W-box required for WRKY TF binding. However, the *G8H* promoter was also found to possess a W-box, and its expression pattern is significantly different to that of *WRKY1*, suggesting that other WRKY TFs could regulate its expression.

4 Conclusions

This research selected and evaluated the stability of ten candidate housekeeping genes for RT-qPCR normalization under three experimental treatments in *U. rhynchophylla* using tissue culture derived seedling leaves. Based on our analysis, *SAM* is the optimal housekeeping gene for normalizing expression levels in *U. rhynchophylla* under our experimental conditions and is potentially an excellent gene for normalization for other RT-qPCR experiments in *U. rhynchophylla*. Using *SAM* as a housekeeping gene in RT-qPCR analysis of the WRKY1 transcription factor and 14 genes associated with TIA biosynthesis showed that the expression pattern of *TDC* and *SGD* is similar to *WRKY1*, and so be involved in regulating these genes.

5 Methods

5.1 Plant material and stress treatment

Seeds of *U. rhynchophylla* plants (deposition number:10410267) were obtained from Guangxi Medicinal Botanical Garden. The tips of *U. rhynchophylla* with similar stem sizes (3 cm) were cut from plants growing in the medicinal plant garden scientific research base, Nanning City, Guangxi Province, China (108.19E;22.49N). We had got the permission to collect *U. rhynchophylla*. The materials were preserved in the germplasm resource nursery of the Academy of Horticulture, Hunan Agricultural University, and identified by Prof. Shugen wei. For abiotic and hormone treatments, tissue culture seedlings with nearly 15 leaves that have been growing for 60 days in tissue culture vessel. Tissue culture seedlings were placed in a chamber with a mean temperature of $25.0 \pm 1.0^{\circ}\text{C}$, relative humidity of $60\% \pm 10\%$, and a day/light cycle of 12/12 h. *U. rhynchophylla* seedlings were subjected to three experimental treatments following the methods as reported [48, 49]. A total of 12 equally growing plants were divided into four groups, including three experimental groups, low temperature, ethylene (ETH), and methyl jasmonate (MeJA) treated leaves and control group (distilled water-treated leaves). For the hormone treatments, the plants were subjected to 100 μM MeJA or 100 μM ETH for 0, 1, 2, 4, 8, 24 h. MeJA and ETH were customized and purchased from Colaber (Beijing, China). For cold stress, tissue culture seedlings were placed in a chamber at 4°C respectively for 0, 4, 8, 12, 24, 48 h. The control group had no treatments but was watered with distilled water over time. All groups had three biological replicates. The clipped leaves were washed with sterilized water, blotted dry, and then snap frozen in liquid nitrogen and stored in -80°C freezer.

5.2 Total RNA extraction and cDNA synthesis

Samples consisting of 50–100 mg of tissue in liquid nitrogen, were fully milled to a fine powder and total RNA extracted using the *SteadyPure* Plant RNA extraction Kit (Accurate Biology, Hunan, China). The purity and concentration of RNA was measured using a Micro Drop (BIO-DL, Shanghai, China), and the RNA integrity was checked on 1% agarose gels. Total RNA (500 ng) was used for reverse transcription with *Evo M-MLV* RT Mix Kit with gDNA Clean (Accurate Biology, Hunan, China) to remove genomic DNA contamination, for qPCR in a 20 μL volume according to the manufacturer's instructions.

5.3 Selection of candidate genes, Primer design and RT-qPCR conditions

Ten candidate house-keeping genes were selected from the full-length genome database obtained using SMRT (Single-Molecule Real-Time) technology on the Oxford Nanopore PromethION (Unpublished, Benagen, Wuhan, China). Specifically, the data used for analysis was based on "Genome + Full-length transcriptome + Metabolomics" co-expression analysis, screening upstream pathway genes in the alkaloid biosynthesis pathway of *U. rhynchophylla*. Specific primer pairs were designed using Beacon Designer 7 software and restricted to primer sequences of 18–25 bp, amplicon length of 80–150 bp, melting temperature (T_m) of $55\text{--}60^{\circ}\text{C}$ and GC content of 45–55%. The specificity of all primers was measured by dissociation curves. The cDNA template stock solution of the samples under different treatments was mixed in equal amounts and diluted 5 times, setting 5 concentration gradients, which were 5^0 , 5^{-1} , 5^{-2} , 5^{-3} and 5^{-4} times the template stock solution. The C_t values of each house-keeping gene at different gradients were obtained by qRT-PCR reaction, and standard curves were generated to calculate the slope (k), correlation coefficient (R^2), and the amplification efficiency of the housekeeping gene using the formula $E = (10^{-1/k}) \times 100\%$. The reaction was carried out in 96-well plates with a Thermal Cycler Dice™ Real Time System III (Takara, Japan). 20 μL reaction system: 2 $\times$ SYBR Green Pro Taq HS premix 10 μL (Vazyme, China), cDNA 2 μL , forward primer and reverse primer 0.4 μL each, RNase free water 7.2 μL . The amplification reaction procedure was: 95°C , 30 s; 95°C , 5 s; 60°C , 30 s; 40 cycles. Each RT-qPCR analysis was performed with three technical replicates and three biological replications.

5.4 Data analysis of housekeeping genes expression stability

The GraphPad Prism 9 software was used to draw the box plots of the distribution of the cycle threshold (C_t) of the ten candidate housekeeping genes in all samples. Three statistical programs, GeNorm, NormFinder, Bestkeeper and one algorithm, Delta C_t were used to evaluate the stability of the candidate housekeeping genes in all experiment treatments. Before using GeNorm and Normfinder software, the C_t values were converted to Q values [50] for

subsequent analysis. The transformation formula used was: $Q = 2^{Ct^{min} - Ct^{sample}}$ (Ct^{sample} is the Ct values of the candidate housekeeping gene in different experimental groups and Ct^{min} is the minimum Ct values of the candidate housekeeping gene in all experimental groups). Bestkeeper and Delta Ct could perform computational analysis directly from the Ct values of candidate genes. The difference is that it determines the stability of the housekeeping genes based on the coefficient of variation (CV) and the standard deviation (SD) of the Ct values. Lower SD and CV values represent the higher gene stability. Based on the results of the above software program, RefFinder (<http://blooge.cn/RefFinder/>) was used to perform a comprehensive stability ranking analysis. The expression of WRKY1 transcription factors and key enzyme genes in the upstream pathways of TIAs synthesis (indole pathway and iridoid pathway) was analyzed in three stress treatments of *U. rhynchophylla* based on the selected housekeeping gene, and the change pattern of relative expression of each key enzyme gene under different treatments was performed using the GraphPad Prism 9 software.

Abbreviations

GES:Geraniol synthase;G8H:Geraniol 8-hydroxylase;8-HGO:8-hydroxygeraniol dehydrogenase;7-DLS:7-deoxyloganetic acid synthase; 7-DLGT:7-deoxyloganetic acid glucosyltransferase;7-DLH:7-deoxyloganic acid 7-hydroxylase; SLS:Secologanin synthase; SGD:Strictosidine- β -D-glucosidase; STR:Strictosidine synthase;AS:Anthranilate synthase;AnPRT:Anthranilate phosphoribosyltransferase; PRAI:Phosphoribosylanthranilate isomerase;TSA:Tryptophan synthase alpha chain;TDC:Tryptophan decarboxylase;TIA:Tepenoid indole alkaloid.

Declarations

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available in NCBI resitory.[*GES*:OP669346,*G8H*:OP669347,*8-HGO*:OP669348,*7-DLS*:OP669349,*7-DLGT*:OP669350,*7-DLH*:OP669351,*SLS*:OP669352,*SGD*:OP669353,*STR*:OP669354,*AS*:OP669355,*AnPRT*:OP669356,*PRAI*:OP669357,*TSA*:OP669358,*TDC*:OP669359,*WRKY1*:OP669360]. Supplement dataset 1 supporting the conclusions and description of a complete protocol are included within the article. The gene sequence information mentioned in this study could be found from the literature based on the gene list in Supplement dataset 2. We had got the permission to collect *U. rhynchophylla*. The plant material collection was in full compliance with relevant institutions,as well as national and international norms and legislation.

Ethics approval and consent to participate

All methods were in compliance with relevant institutional,national,and international guidelines and legislation.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DTM conceived the study, participated in experiment and data analysis, and drafted the manuscript. YYS and JLH participated in experiment and data analysis. QT and YL initiated the project, helped to conceive the study, revised the manuscript and participated in the design and coordination. LNZ participated in experiment materials preparation. DYQ and IWW participated in data analysis and manuscript revision. All authors read and approved the final manuscript.

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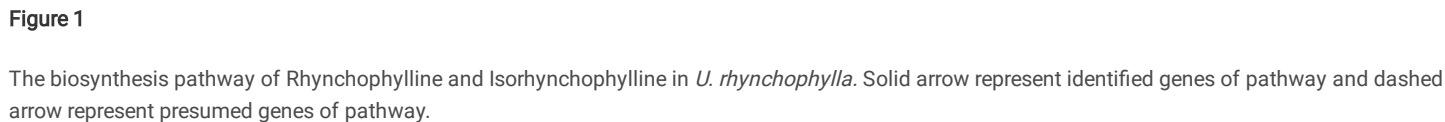
References

1. Ling LZ, Zhang SD. The complete chloroplast genome of the traditional Chinese herb, *Uncaria rhynchophylla* (Rubiaceae). Mitochondrial DNA B Resour. 2020;5:424-5.

2. Loh YC, Ch'ng YS, Tan CS, Ahmad M, Asmawi MZ, Yam MF. Mechanisms of action of *Uncaria rhynchophylla* ethanolic extract for its vasodilatory effects. *J Med Food*. 2017;20:895-911.
3. Pan H, Yao C, Yang W, Yao S, Huang Y, Zhang Y, et al. An enhanced strategy integrating offline two-dimensional separation and step-wise precursor ion list-based raster-mass defect filter: characterization of indole alkaloids in five botanical origins of *Uncariae Ramulus Cum Unicis* as an exemplary application. *J Chromatogr A*. 2018;1563:124-34.
4. Li RF, Guo QL, Zhu CG, Xu CB, Wei YZ, Cai J, et al. Minor triterpenes from an aqueous extract of the hook-bearing stem of *Uncaria rhynchophylla*. *J Asian Nat Prod Res*. 2021;23:307-17.
5. Li R, Cheng J, Jiao M, Li L, Guo C, Chen S, et al. New phenylpropanoid-substituted flavan-3-ols and flavonols from the leaves of *Uncaria rhynchophylla*. *Fitoterapia*. 2017;116:17-23.
6. Zeng P, Wang XM, Ye CY, Su HF, Tian Q. The main alkaloids in *Uncaria rhynchophylla* and their anti-Alzheimer's disease mechanism determined by a network pharmacology approach. *Int J Mol Sci*. 2021;22:3612.
7. Soltani N, Nazarian-Firouzabadi F, Shafeinia A, Sadr AS, Shirali M. The expression of Terpenoid Indole Alkaloid (TIAs) pathway genes in *Catharanthus roseus* in response to salicylic acid treatment. *Mol Biol Rep*. 2020;47:7009-16.
8. Thakur M, Sohal BS. Role of elicitors in inducing resistance in plants against pathogen infection: a review. *ISRN Biochem*. 2013;2013:762412.
9. Kumar P, Pal T, Sharma N, Kumar V, Sood H, Chauhan RS. Expression analysis of biosynthetic pathway genes vis-à-vis podophyllotoxin content in *Podophyllum hexandrum* royle. *Protoplasma*. 2015;252:1253-62.
10. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol*. 2002;29:23-39.
11. Chen R, Gyokusen M, Nakazawa Y, Gyokusen K. Selection of housekeeping genes for transgene expression analysis in *Eucommia ulmoides* oliver using real-time RT-PCR. *J Bot*. 2010;2010:230961.
12. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3:Research0034.
13. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol*. 2000;25:169-93.
14. Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, et al. Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics*. 2004;18:226-31.
15. Maroufi A, Van Bockstaele E, De Loose M. Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. *BMC Mol Biol*. 2010;11:15.
16. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol*. 2005;139:5-17.
17. Remans T, Smeets K, Odenakker K, Mathijsen D, Vangronsveld J, Cuypers A. Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. *Planta*. 2008;227:1343-9.
18. Suttipanta N, Pattanaik S, Kulshrestha M, Patra B, Singh SK, Yuan L. The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. *Plant Physiol*. 2011;157:2081-93.
19. Tang Q, Ma X, Mo C, Wilson IW, Song C, Zhao H, et al. An efficient approach to finding *Siraitia grosvenorii* triterpene biosynthetic genes by RNA-seq and digital gene expression analysis. *BMC Genomics*. 2011;12:343.
20. Dai L, Liu C, Zhu Y, Zhang J, Men Y, Zeng Y, et al. Functional characterization of cucurbitadienol synthase and triterpene glycosyltransferase involved in biosynthesis of mogrosides from *Siraitia grosvenorii*. *Plant Cell Physiol*. 2015;56:1172-82.
21. Zhang J, Dai L, Yang J, Liu C, Men Y, Zeng Y, et al. Oxidation of cucurbitadienol catalyzed by CYP87D18 in the biosynthesis of mogrosides from *Siraitia grosvenorii*. *Plant Cell Physiol*. 2016;57:1000-7.
22. Yang W, Ip SP, Liu L, Xian YF, Lin ZX. *Uncaria rhynchophylla* and its major constituents on central nervous system: a review on their pharmacological actions. *Curr Vasc Pharmacol*. 2020;18:346-57.
23. Zhang JG, Geng CA, Huang XY, Chen XL, Ma YB, Zhang XM, et al. Chemical and biological comparison of different sections of *Uncaria rhynchophylla* (Gou-Teng). *Eur J Mass Spectrom (Chichester)*. 2017;23:11-21.
24. Guo Q, Ma X, Wei S, Qiu D, Wilson IW, Wu P, et al. De novo transcriptome sequencing and digital gene expression analysis predict biosynthetic pathway of rhynchophylline and isorhynchophylline from *Uncaria rhynchophylla*, a non-model plant with potent anti-Alzheimer's properties. *BMC Genomics*. 2014;15:676.
25. Wang JJ, Ji LL, Deng XH, Lyu LT, Yu LF, Guan P. Effects of light intensity on growth and content of active components of *Uncaria rhynchophyll*. *Zhongguo Zhong Yao Za Zhi*. 2019;44:5118-23.
26. Ashihara H, Kato M, Crozier A. Distribution, biosynthesis and catabolism of methylxanthines in plants. *Handb Exp Pharmacol*. 2011;200:11-31.
27. Albertos P, Wagner K, Poppenberger B. Cold stress signalling in female reproductive tissues. *Plant Cell Environ*. 2019;42:846-53.
28. Kozera B, Rapacz M. Reference genes in real-time PCR. *J Appl Genet*. 2013;54:391-406.
29. Wang H, Cai QZ, Liu L, Yang Q, Zhou LY. Reference gene screening for real-time quantitative PCR in *Polygonum multiflorum*. *Zhongguo Zhong Yao Za Zhi*. 2021;46:80-5.
30. De Sousa FCB, Do Nascimento CS, Macário MDS, Araújo RDS, Barbosa LT, Bayão GFV, et al. Selection of reference genes for quantitative real-time PCR normalization in European quail tissues. *Mol Biol Rep*. 2021;48:67-76.

31. Shukla P, Reddy RA, Ponnuvel KM, Rohela GK, Shabnam AA, Ghosh MK, et al. Selection of suitable reference genes for quantitative real-time PCR gene expression analysis in Mulberry (*Morus alba* L.) under different abiotic stresses. *Mol Biol Rep.* 2019;46:1809-17.
32. Mallona I, Lischewski S, Weiss J, Hause B, Egea-Cortines M. Validation of reference genes for quantitative real-time PCR during leaf and flower development in *Petunia hybrida*. *BMC Plant Biol.* 2010;10:4.
33. Hong SY, Seo PJ, Yang MS, Xiang F, Park CM. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real-time PCR. *BMC Plant Biol.* 2008;8:112.
34. Huang L, Yan H, Jiang X, Zhang Y, Zhang X, Ji Y, et al. Reference gene selection for quantitative real-time reverse-transcriptase PCR in orchardgrass subjected to various abiotic stresses. *Gene.* 2014;553:158-65.
35. Cai J, Li P, Luo X, Chang T, Li J, Zhao Y, et al. Selection of appropriate reference genes for the detection of rhythmic gene expression via quantitative real-time PCR in Tibetan hulless barley. *PLoS One.* 2018;13:e0190559.
36. Huang Y, Tan H, Yu J, Chen Y, Guo Z, Wang G, et al. Stable internal reference genes for normalizing real-time quantitative PCR in *Baphicacanthus cusia* under hormonal stimuli and UV irradiation, and in different plant organs. *Front Plant Sci.* 2017;8:668.
37. Screening of qRT-PCR internal reference genes for four leaf color phenotypes of *Sinobambusa tootsik* f. *luteoloalbostrata*. *Mol Plant Breed.* 2019;14:4592-4599.
38. Wang G, Tian C, Wang Y, Wan F, Hu L, Xiong A, et al. Selection of reliable reference genes for quantitative RT-PCR in garlic under salt stress. *PeerJ.* 2019;7:e7319.
39. Sun H, Jiang X, Sun M, Cong H, Qiao F. Evaluation of reference genes for normalizing RT-qPCR in leaves and suspension cells of *Cephalotaxus hainanensis* under various stimuli. *Plant Methods.* 2019;15:31.
40. De Spiegelaere W, Derr-Wieloch J, Weigel R, Schumacher V, Schorle H, Nettersheim D, et al. Reference gene validation for RT-qPCR, a note on different available software packages. *PLoS One.* 2015;10:e0122515.
41. De Carvalho K, Filho JCB, Dos Santos TB, De Souza SG, Vieira LG, Pereira LF, et al. Nitrogen starvation, salt and heat stress in coffee (*Coffea arabica* L.): identification and validation of new genes for qPCR normalization. *Mol Biotechnol.* 2013;53:315-25.
42. Couto CC, Santos TF, Mamede A, Oliveira TC, Souza AM, Freitas-Silva O, et al. *Coffea arabica* and *C. canephora* discrimination in roasted and ground coffee from reference material candidates by real-time PCR. *Food Res Int.* 2019;115:227-33.
43. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 2004;64:5245-50.
44. Mao M, Xue Y, He Y, Zhou X, Hu H, Liu J, et al. Validation of reference genes for quantitative real-time PCR normalization in *Ananas comosus* var. *bracteatus* during chimeric leaf development and response to hormone stimuli. *Front Genet.* 2021;12:716137.
45. Li G, Ma J, Yin J, Guo F, Xi K, Yang P, et al. Identification of reference genes for reverse transcription-quantitative PCR analysis of ginger under abiotic stress and for postharvest biology studies. *Front Plant Sci.* 2022;13:893495.
46. Roepke J, Salim V, Wu M, Thamm AM, Murata J, Ploss K, et al. Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. *Proc Natl Acad Sci U S A.* 2010;107:15287-92.
47. Vom Endt D, Kijne JW, Memelink J. Transcription factors controlling plant secondary metabolism: what regulates the regulators? *Phytochemistry.* 2002;61:107-14.
48. Li T, Wang J, Lu M, Zhang T, Qu X, Wang Z. Selection and validation of appropriate reference genes for qRT-PCR analysis in *Isatis indigotica* fort. *Front Plant Sci.* 2017;8:1139.
49. Wang Z, Xu J, Liu Y, Chen J, Lin H, Huang Y, et al. Selection and validation of appropriate reference genes for real-time quantitative PCR analysis in *Momordica charantia*. *Phytochemistry.* 2019;164:1-11.
50. Wang Q, Ishikawa T, Michiue T, Zhu BL, Guan DW, Maeda H. Stability of endogenous reference genes in postmortem human brains for normalization of quantitative real-time PCR data: comprehensive evaluation using geNorm, NormFinder, and BestKeeper. *Int J Legal Med.* 2012;126:943-52.

Figures



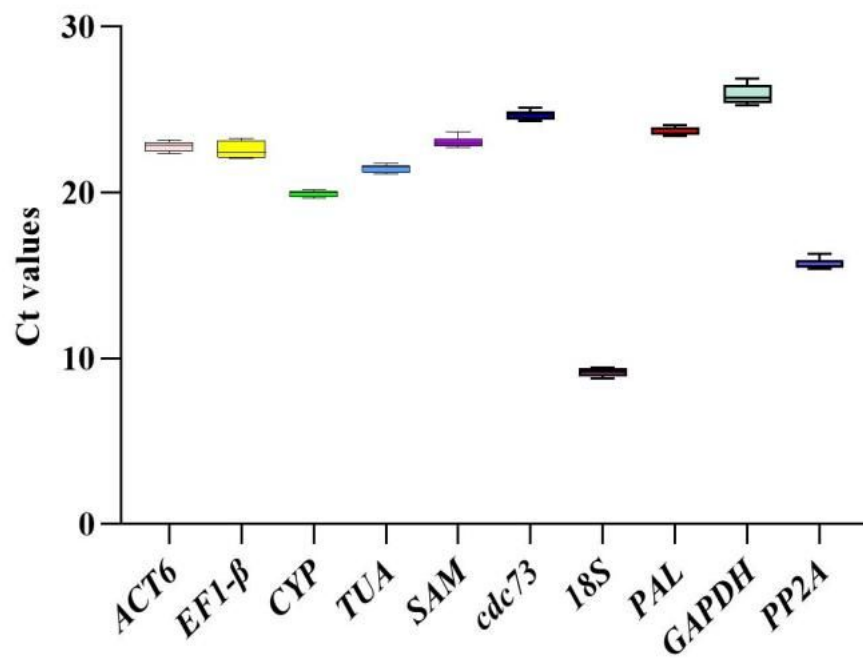


Figure 2

The Ct value of 10 candidate housekeeping genes in all samples. The expression data is displayed as the Ct value of each housekeeping gene in the sample of *U. rhynchophylla*. Boxplot indicate the 25th/75th percentiles and it contains the mean, maximum, minimum values.

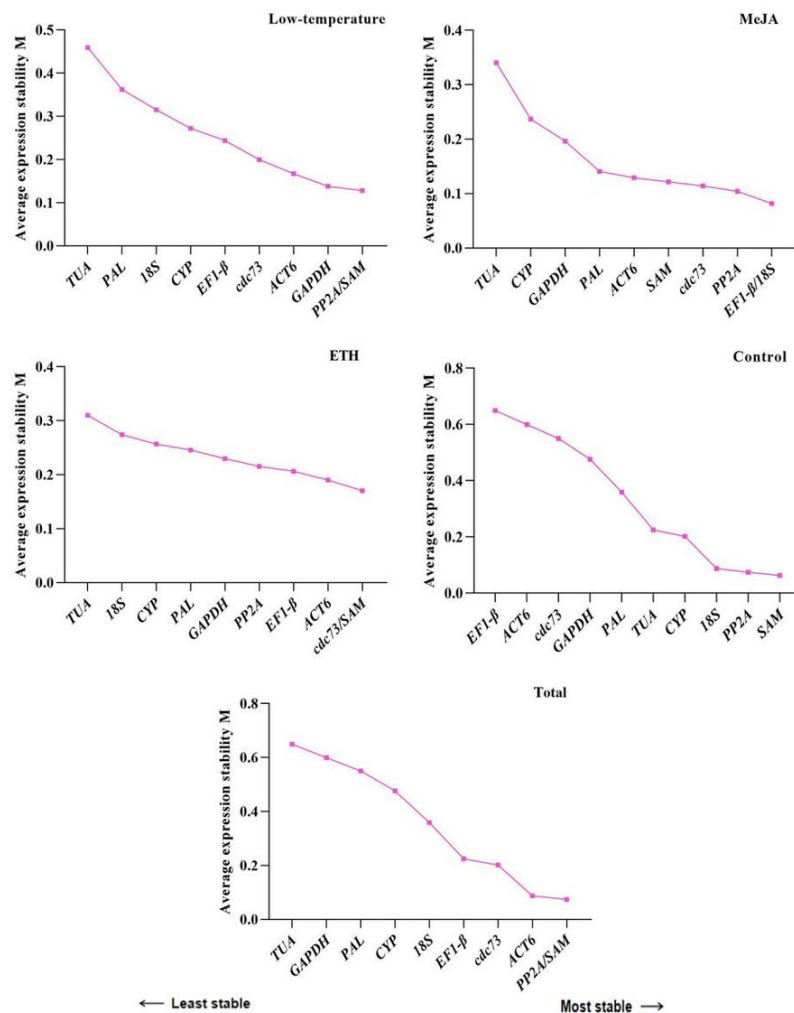


Figure 3

Average expression stability values (M) of ten candidate housekeeping genes using GeNorm. Expression stability was evaluated in samples from leaves of *U. rhynchophylla* was subjected to low temperature, MeJA (100 μ mol), ETH (100 μ mol), control (not treated) and total (all treated samples). The least stable genes are on the left with higher M value and the most stable genes on the right with lower M value.

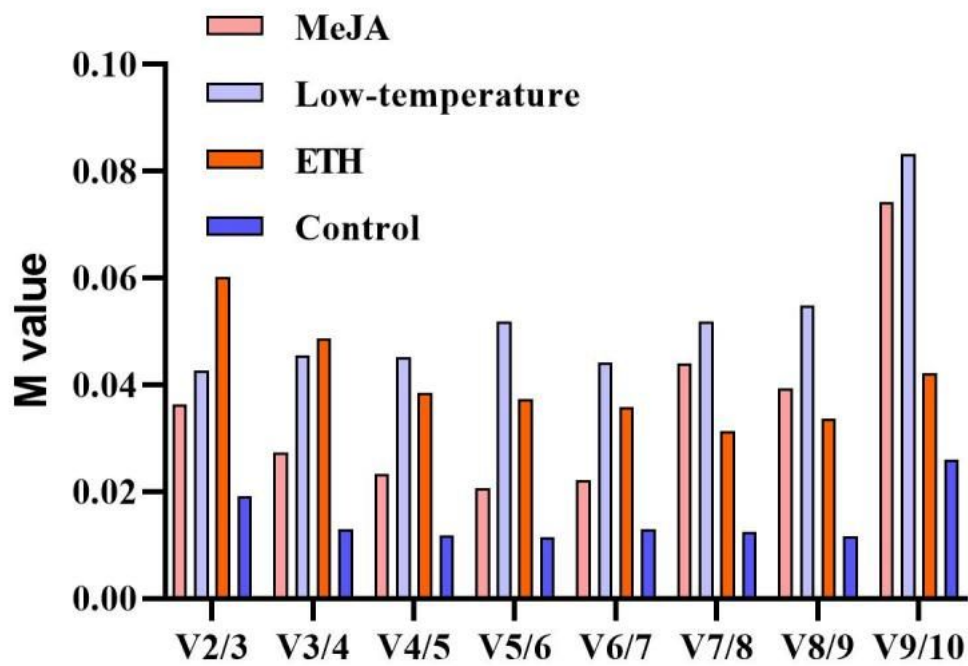


Figure 4

The pairwise variation ($V_n/n+1$) scores of ten candidates measured by GeNorm. Different treatments are identified by different colors. The value to determine the appropriate number of the housekeeping genes for RT-qPCR normalization is 0.15.

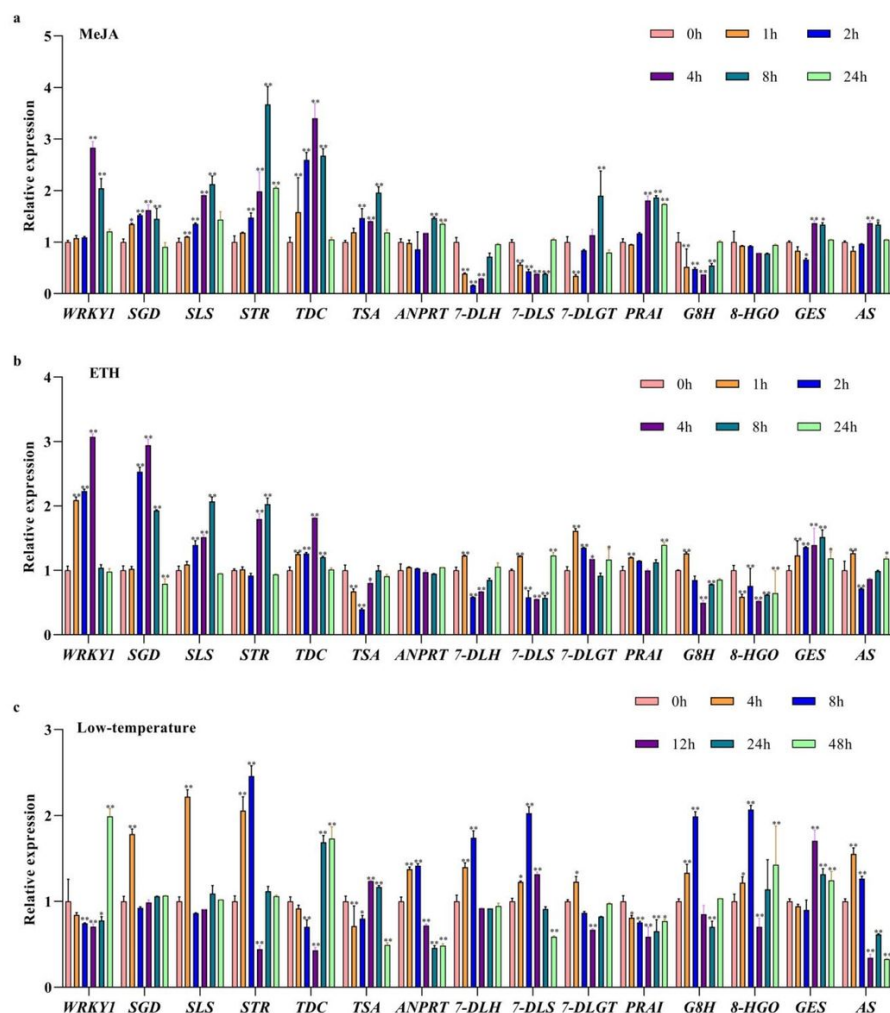


Figure 5

The relative expression of WRKY1 transcription factor and key enzyme genes in response to MeJA, ETH and low temperature. Different color represents different times. (a) MeJA treatment; (b) Ethylene treatment; (c) Low temperature treatment. The error bars represent the means $\pm$ SD from three biological replicates, and asterisks indicate statistically significant differences compared with the controls (0h). * $P < 0.05$; ** $P < 0.01$.

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